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Atomic force microscopy study on specificity and non-specificity of interaction forces between *Enterococcus faecalis* cells with and without aggregation substance

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Enterococcus faecalis is one of the leading causes of hospital-acquired infections, and indwelling medical devices are especially prone to infection. *E. faecalis* expressing aggregation substance (Agg) adheres to biomaterial surfaces by means of positive cooperativity, i.e. the ability of one adhering organism to stimulate adhesion of other organisms in its immediate vicinity. In this study, atomic force microscopy (AFM) was used to measure the specificity and non-specificity of interaction forces between *E. faecalis* cells with and without Agg. Bacteria were attached to a substratum surface and a tip-less cantilever. Two *E. faecalis* strains expressing different forms of Agg showed nearly twofold higher interaction forces between bacterial cells than a strain lacking Agg [adhesive force (F_{adh}), −1·3 nN]. The strong interaction forces between the strains with Agg were reduced after adsorption of antibodies against Agg from −2·6 and −2·3 nN to −1·2 and −1·3 nN, respectively. This suggests that the non-specific interaction force between the enterococci amounts to approximately 1·2 nN, while the specific force component is only twofold stronger. Comparison of the results of the AFM interaction forces with the positive cooperativity after adhesion to a biomaterial in a parallel-plate flow chamber showed that in the absence of strong interaction forces between the cells, positive cooperativity was also absent. In conclusion, this is believed to be the first time that the influence of specific antibodies on interaction forces between *E. faecalis* cells has been demonstrated by AFM, thereby experimentally distinguishing between specific and non-specific force components.

INTRODUCTION

Enterococci are becoming one of the leading causes of hospital-acquired infections (Richards *et al.*, 2000), with *Enterococcus faecalis* accounting for up to 90 % of clinical enterococcal isolates (Ruoff *et al.*, 1990). Indwelling medical devices are a frequent source of these enterococcal infections (Dickinson & Bisno, 1989; Yu *et al.*, 1996). A bacterial biofilm attached to the indwelling device can be a source of persistent infection. Microbial adhesion and aggregation are crucial steps in biofilm formation, and therefore in biomaterial-centred infections (Costerton *et al.*, 1999).

Aggregation substance (Agg) is a plasmid-encoded surface protein of *E. faecalis*, and it is associated with infection, aggregation and biofilm formation (Jett *et al.*, 1994; Waar *et al.*, 2002a, b). Two different forms of Agg have been described: Asa1 and Asa373 (De Boever *et al.*, 2000; Galli *et al.*, 1990). Previously, we reported that *E. faecalis* expressing Agg adheres in significantly higher numbers to biomaterials compared with isogenic strains without Agg. Adhesion was studied in a parallel-plate flow chamber, and the increase in adhesion was through specific interaction between the bacteria on the surface mediated by the Agg (Waar *et al.*, 2002b). This specific interaction was expressed as the degree of positive cooperativity, i.e. the ability of one adhering organism to stimulate the adhesion of other organisms in its immediate vicinity. Positive cooperativity is directly reflected in the spatial arrangement of adhering organisms over a substratum surface, and is concluded from the high local relative densities around a given adhering organism (Sjollema & Busscher, 1990).

In the past, specific interactions between biological surfaces have been opposed to non-specific interactions.

Abbreviations: AFM, atomic force microscopy; Agg, aggregation substance; FEP, fluoro-ethylene-propylene.

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Specific interactions are frequently described in terms of stereochemical interactions between localized complementary molecular groups, and sometimes even in terms of specific forces, as being a separate class of fundamental interaction forces. However, it is important to realize that all interaction forces originate from the same fundamental forces (Van Oss, 1991), including the ever-present Lifshitz–Van der Waals forces, electrostatic forces, hydrogen bonding and Brownian motion forces, and specific interaction forces only distinguish themselves by being highly directional and spatially confined (Busscher *et al.*, 1992). Alternatively, non-specific interactions arise from interaction forces between all molecules of the entire cell and substratum, and are consequently of a more long-range character, without being directional or spatially confined. Overall, long-range and non-specific fundamental interaction forces and short-range, specific interactions operate in concert, and hitherto have never been individually assessed on an experimental basis.

Atomic force microscopy (AFM) is a surface imaging technique, which operates by sensing the force between a very sharp probe attached to a flexible cantilever and the sample surface (Binnig *et al.*, 1986). Recently, AFM has emerged as a powerful tool to measure molecular interaction forces (Dufrêne, 2003). AFM force measurements have been further applied to microbial systems, measuring the interaction between bacteria and a substratum surface (Bowen *et al.*, 2002; Lower *et al.*, 2001; Razatos *et al.*, 1998). One approach to study these microbial interactions is to attach the bacteria directly onto the AFM probe or cantilever, and study the interaction with the substratum. However, insight into the process of microbial aggregation in biofilm formation would require the investigation of the direct interaction between two bacteria, which has hitherto not been done, as this is experimentally very difficult. However, if bacteria are attached to both the cantilever and a substratum surface, it should be possible to study the interaction forces between bacteria by AFM.

Here, we report the use of AFM force measurements to study the specificity and non-specificity of the interaction between *E. faecalis* strains expressing either Asa1 or Asa373 by attaching the bacteria to both a substratum surface and a tip-less cantilever. The results were compared with the positive cooperativity after adhesion to poly(tetrafluoroethylene-*co*-hexafluoropropylene) (fluoro-ethylene-propylene, FEP), a frequently used biomaterial, in a parallel-plate flow chamber. The role of Agg in the specific interaction component and in biofilm formation was confirmed by incubating the bacteria with antibodies specific for Asa1 or Asa373 prior to both the AFM force measurements and adhesion in the parallel-plate flow chamber. Insight into the mechanism of direct interaction between enterococci, the role of Agg in this interaction, and the influence of specific antibodies on this interaction, might lead to ways to prevent biofilm formation by enterococci on indwelling medical devices.

METHODS

Bacterial strains, culture conditions and harvesting. Three isogenic *E. faecalis* strains were used in this study: the plasmid-free strain OG1X (Ike *et al.*, 1983); OG1X containing the sex-pheromone-responsive plasmid pAD1 encoding the Agg Asa1, with a positive regulator gene inserted, which induces constitutive expression of this plasmid (depicted as OG1XE : pAD1, where E indicates the positive regulator gene) (Muscholl *et al.*, 1993); and OG1X containing the plasmid pAM373, which expresses Asa373 after induction with pheromones (depicted as OG1X : pAM373) (Clewell *et al.*, 1985). Expression of the Agg was checked by immunofluorescence with polyclonal antibodies against Asa1 or Asa373. A similar level of Agg expression on both Agg-positive strains was detected (data not shown). A. B. Muscholl-Silberhorn, Thetis-IBN, Hamburg, Germany, kindly provided OG1X strains and polyclonal antisera.

The strains were streaked and grown overnight at 37 °C from a frozen stock on blood agar. Several colonies were used to inoculate 3 ml Todd–Hewitt broth (THB; Oxoid) that was incubated at 37 °C in ambient air for 24 h. From this preculture, 2 ml was used to inoculate a second culture of 200 ml THB that was grown for 18 h. If necessary, bacteria from the second culture were incubated with polyclonal antiserum (1 : 600) for 30 min at 37 °C. In a pilot study, different dilutions of serum were tested in the parallel-plate chamber, and a dose–effect relation was observed; from these experiments the dilution of 1 : 600 was chosen to be used in this study. Bacteria were harvested by centrifugation at 10 000 ***g*** for 5 min at 10 °C, and washed twice with demineralized water. Subsequently, bacteria were suspended in PBS (10 mM potassium phosphate, 0·15 M NaCl, pH 7), and sonicated on ice for 2×10 s to separate cell clusters. For the parallel-plate flow chamber experiments, bacteria were counted in a Bürker-Türk counting chamber, and diluted to a concentration of 3×10^8 cells ml^{-1}.

E. faecalis JH2-2 excretes all known sex pheromones of *E. faecalis* into the growth medium, and it was used to collect pheromones (Jacob & Hobbs, 1974). After 24 h growth at 37 °C in THB, the culture was centrifuged at 10 000 ***g*** for 10 min at 10 °C, and the supernatant containing the pheromones was autoclaved. To induce the expression of Agg in strain OG1X : pAM373, growth in the presence of pheromone is necessary; therefore, the second culture of strain OG1X : pAM373 consisted of 100 ml fresh THB, and 100 ml pheromone-containing THB supernatant.

Polyclonal antibodies. Purified Asa1 and Asa373 were used for the production of polyclonal antisera. The genes encoding either Asa1 or Asa373 were constructed in vector pQE30-32 (Qiagen), expressed in *Escherichia coli* cloning strain JM109, and purified as described by Muscholl-Silberhorn (1998, 1999). Eurogentec carried out immunization according to a standardized procedure (injections on days 0, 14, 28 and 56, and bleeds on days 0, 38 and 64). Prior to immunization, serum was tested for absence of cross-reactivity with aggregation substances or unrelated proteins from *E. faecalis* and *Escherichia. coli.* The polyclonal antisera were tested for specificity for Asa1 or Asa373 using Western blots with purified Agg, and crude protein preparations of enterococci expressing different forms of Agg.

AFM. Bacteria were attached through electrostatic interactions (physical adsorption) to both a glass slide and a tip-less cantilever, made positively charged through adsorption of poly L-lysine (Bolshakova *et al.*, 2001). In order to coat the glass surface with poly L-lysine, the glass slide was cleaned by dipping in methanol, and rinsing with demineralized water, after which a drop of 0·01 % (w/v) poly L-lysine (Sigma) solution was added, and spread over the surface. After air-drying of the glass slide, a few drops of the undiluted bacterial suspension in PBS were added. After 15 min, the

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bacteria-coated slide was rinsed with PBS to wash off the bacterial suspension, and transferred to the AFM. The tip-less AFM cantilever (NP-0; Veeco) was dipped into a drop of 0·01 % (w/v) poly L-lysine solution, and allowed to dry; afterwards, the cantilever was dipped into a drop of bacterial suspension, and dried again.

AFM force–distance measurements were made at room temperature under PBS using a Dimension 3100 system (Nanoscope III; Digital Instruments). 'V'-shaped tip-less silicon nitride cantilevers from Veeco, with a spring constant of 0·06 N m^{-1}, were used. Individual force curves were collected between the bacteria-coated AFM cantilever and the top of randomly selected physically adsorbed bacteria, with *z*-displacement of 1000–2000 nm at *z*-scan rates $\leqslant$1 Hz. The slope of the retraction force curves in the region where probe and sample were in contact was used to convert the voltage into cantilever deflection. The conversion of deflection into force was carried out as described by Dufrêne (2000). Approach curves were fitted to an exponential function. Retraction curves generally showed multiple adhesion peaks, and the magnitude of the peaks was recorded and averaged. Results represent the mean of two separate runs, with a total of 30 force–distance curves taken from six different bacteria (five curves per bacterium). The results were further normalized with respect to the mean of both runs.

Parallel-plate flow chamber, image analysis and adhesion. The flow chamber (internal dimensions: length × width × height, 76 × 38 × 0·6 mm) and image analysis system have been described in detail previously (Busscher & Van der Mei, 1995). FEP was obtained from Fluorplast. Images were taken from the Perspex-bottom plate (58 × 38 mm) of the parallel-plate flow chamber, which was completely covered with FEP. Surfaces were sonicated for 3 min in a surfactant solution (2 % RBS 35 detergent in water; Omniclean), rinsed thoroughly with water, and then washed with methanol and demineralized water before use. The flow chamber was cleaned with Extran (Merck), and thoroughly rinsed with water and demineralized water. Prior to each experiment, all tubes and the flow chamber were filled with PBS, taking care to remove all air bubbles from the system. Once the system was filled, a bacterial suspension of 3×10^8 cells ml^{-1} in PBS was allowed to flow through the system at a flow rate of 1·44 ml min^{-1}, corresponding to a shear rate of 10·6 s^{-1}. Deposition was observed with a CCD-MXRi camera (High Technology) mounted on a phase-contrast microscope (Olympus BH-2) equipped with a ×40 ultra-long-working-distance lens (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyser (TEA; Difa). The bacterial suspension was perfused through the system for 4 h with recirculation at room temperature, and images were taken at different time intervals and analysed. Adhesion experiments were performed in triplicate with separate bacterial cultures.

RESULTS

The interaction forces measured between the *E. faecalis* cells are plotted as a function of the separation distance in Fig. 1. *E. faecalis* strain OG1X showed similar force–distance curves with and without incubation with antibodies to Asa1 or Asa373, and therefore only the curve without incubation is shown (Fig. 1a). The force–distance curves of the *E. faecalis* strains expressing Agg (Fig. 1b, d) show high adhesion forces upon retraction over a long distance, probably due to stretching of surface structures comprising the interacting groups, as corroborated by Van der Mei *et al.* (2000) for streptococci. Comparing the force–distance curves of the *E. faecalis* strains expressing Agg (Fig. 1b, d) with the force–distance curves after incubation with specific antibodies reveals a remarkable decrease in adhesion upon retraction (Fig. 1c, e). Approach curves did not show significant differences between the *E. faecalis* strains, and all showed a repulsive force upon contact of about 5·5 nN, with a characteristic decay length of about 64 nm.

After adhesion to FEP in the parallel-plate flow chamber, bacteria per unit area were least-square fitted to an exponential curve, and the number of bacteria adhering at stationary end-point (n at t_∞) could be estimated from this exponential curve. Furthermore, the degree of positive cooperativity between adhering bacteria was assessed by comparing the local densities of bacterial adhesion around all individual bacteria with the mean bacterial density over the substratum surface, yielding the radial pair distribution function $g(r)$ (Waar *et al.*, 2002b). When enterococci are randomly distributed over the entire substratum surface, $g(r) = 1$. However, if there is preferential adhesion at a given separation distance r between adhering bacteria through positive cooperative mechanisms, then $g(r) > 1$.

Table 1 summarizes the interaction forces between the *E. faecalis* cells, their degree of positive cooperativity [$g(r_p)$], and the mean bacterial density on FEP at stationary end-point (n at t_∞). The mean interaction forces upon retraction (F_{adh}) are stronger for the *E. faecalis* strains expressing Agg (OG1XE : pAD1 and OG1X : pAM373) compared with the strain without Agg (OG1X). This is in line with the higher positive cooperativity measured for the strains expressing Agg. The F_{adh} and $g(r_p)$ values decreased remarkably for the *E. faecalis* strains expressing Agg after incubation with the specific antiserum, which indicates specific interference of the antibodies with the interaction between the strains. Interestingly, the number of bacteria at stationary end-point also decreased for the *E. faecalis* strains expressing Agg after incubation with polyclonal antibodies against the Agg. This decrease was not seen for the OG1X strain after incubation with the antibodies, which indicates that the decrease was due to specific interaction with the Agg.

DISCUSSION

In this paper, we show that the interaction forces between bacteria can be measured by use of the AFM force measurements during which the bacteria are attached to both a substratum surface and a tip-less cantilever. The measurement of interaction forces between two bacteria allows less accurate control of the contact area between the interacting surfaces than when, for instance, the AFM tip itself is used to probe a cell surface (Vadillo-Rodriguez *et al.*, 2004). However, since the force values measured in this study between bacteria, and those measured between an AFM tip and bacteria immobilized in membrane filters (Vadillo-Rodriguez *et al.*, 2004), are of the same order of magnitude, it is likely that only one contact point is involved in measuring the enterococcal interaction forces. Furthermore, the limited range of the interaction forces excludes an intervening influence of the positively charged glass surface, as these

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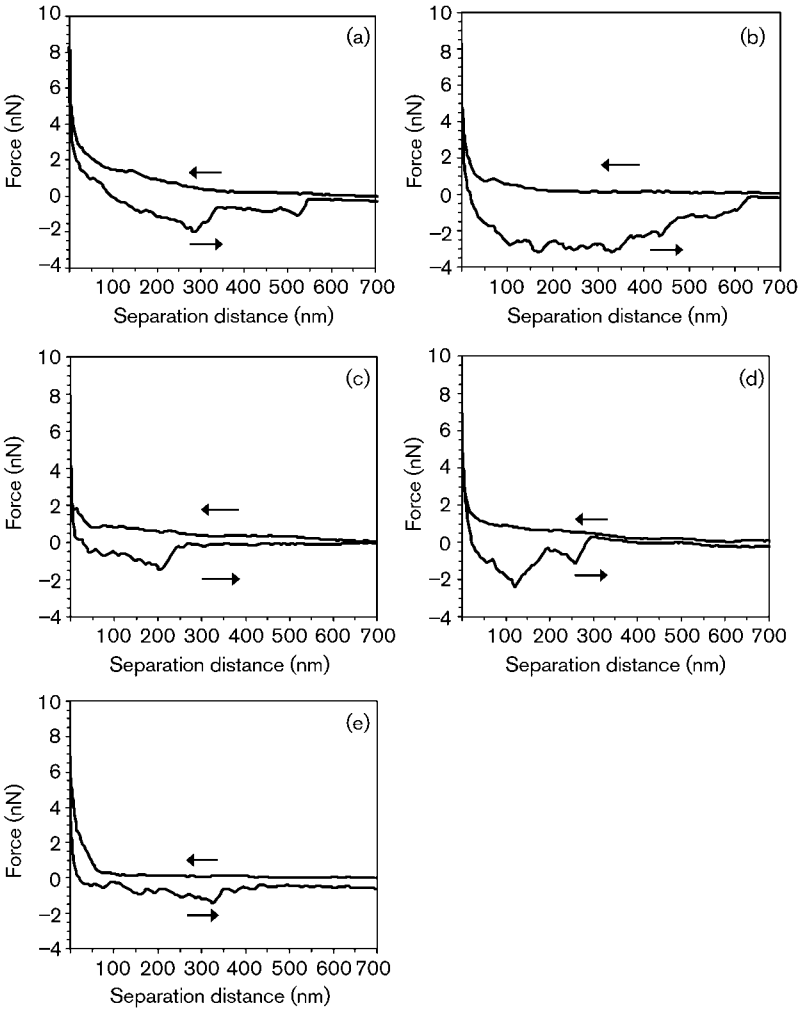

Fig. 1. Representative examples of AFM force–distance curves between *E. faecalis* strains on a silicone nitride tip-less cantilever and a positively charged glass surface. (a) Strain OG1X without Agg, (b) strain OG1XE : pAD1 expressing Asa1, (c) strain OG1XE : pAD1 incubated with antibodies to Asa1, (d) strain OG1X : pAM373 expressing Asa373, and (e) strain OG1X : pAM373 incubated with antibodies to Asa373. The arrows indicate the direction of movement of the cantilever.

forces do not range over the micron-sized diameter of the enterococci, while the strength of the electrostatic interaction between poly L-lysine and the negatively charged enterococci ensures sufficient immobilization. Moreover, the interaction forces between different strains of *E. faecalis*, with and without surface Agg, and after incubation with antibodies to Agg, were found to relate with the positive cooperativity observed after their adhesion to a biomaterial surface.

Microbial aggregation and adhesion are crucial events in the formation of biofilms in medicine and nature. Understanding the individual interaction forces between aggregating bacteria and their specific and non-specific components can give more insight into the molecular basis of these phenomena. Agg is a surface protein of *E. faecalis* that enables close cell–cell aggregation between bacteria, and transfer of plasmids (Dunny *et al.*, 1978). Previously, we showed that Agg enhances the adhesion to biomaterials through positive cooperativity, which is interaction between the bacteria on the biomaterial surface. The adhesion force upon retraction measured with AFM, and the positive cooperativity after adhesion in the parallel-plate flow chamber, showed a good correlation. Therefore it can be concluded that the interaction forces measured with the AFM are relevant for the macroscopic colonization of biomaterial surfaces by *E. faecalis* cells, and that antibodies to Agg can block this interaction, leaving only the non-specific

Table 1. Comparison of AFM interaction forces and adhesion characteristics of *E. faecalis*

Adhesion experiments were performed in triplicate with separate bacterial cultures. AFM data are results of 30 measurements on six different bacteria in two separate runs.

Strain	Pre-incubation	F_{adh}*	$g(r_p)$†	n at t_∞‡
OG1X	No	−1·3±0·5	1·4±0·1	6·1±0·5
OG1X	Anti-Asa1	−1·2±0·5	1·2±0·1	7·1±0·2
OG1X	Anti-Asa373	−1·5±0·4	1·2±0·1	6·9±1·1
OG1XE : pAD1	No	−2·6±0·5	3·2±0·8	14·0±1·1
OG1XE : pAD1	Anti-Asa1	−1·2±0·4	1·9±0·1	10·5±0·8
OG1X : pAM373	No	−2·3±0·6	2·0±0·4	11·5±0·5
OG1X : pAM373	AntiAsa373	−1·3±0·4	1·2±0·1	10·4±0·7

*Mean adhesion force upon retraction measured in nN (±SD).
†Degree of positive cooperativity (±SD).
‡Number of bacteria at stationary end-point ($\times 10^6$ cm^{-2}) (±SD).

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interaction force component operative. For enterococci, this force component can be estimated from the present study to amount to approximately 1·2 nN, which is remarkably still half the interaction force observed for enterococci interacting with a specific force component.

To confirm the role of Agg in the interaction between the *E. faecalis* strains, the bacteria were incubated with antibodies specific for Asa1 or Asa373 prior to AFM force measurements and adhesion experiments. The results showed a clear decrease in both the adhesion force upon retraction and the positive cooperativity, indicating that the interaction was through Agg, and that it could be inhibited with antibodies to the Agg. Non-specific influences of the antibodies were excluded by incubating the strain without Agg (OG1X) with antibodies, but this did not yield a change in interaction forces or positive cooperativity.

After incubation with polyclonal antiserum, the presence of antibodies on the bacteria in suspension was checked by immunofluorescence with FITC-labelled mouse anti-rabbit antibodies (data not shown). The immunofluorescence assay showed antibody coating only for *E. faecalis* expressing Agg when incubated with the matching polyclonal antiserum, which showed that it was the specific antibodies, and not other components of the serum, that interacted with the forces between the *E. faecalis* strains.

Remarkably, not only the positive cooperativity, but also the number of adhering bacteria at stationary end-point, decreased after incubation with antibodies to Agg. A role in the prevention of biomaterial-related enterococcal infections might possibly be assigned to these antibodies because of their interference with positive cooperative mechanisms of adhesion. Other studies performed on the influence of antibodies on bacterial adhesion are in line with our current results, and they showed a decrease in the number of bacteria adhering at the stationary end-point (Van Raamsdonk *et al.*, 1995) or the initial deposition rate (Poelstra *et al.*, 2000). Agg is associated with enterococci causing infections in, or colonizing, hospitalized patients. Here, it is shown that antibodies to Agg, obtained passively, or actively via immunization, could play a role in the prevention of infections with enterococci in hospitalized patients.

In conclusion, this is believed to be the first time that the influence of specific antibodies on interaction forces between *E. faecalis* cells has been demonstrated by AFM. The specific interaction forces can be diminished by adsorption of antibodies specific to Agg, but leave a sizeable non-specific interaction force amounting to approximately half the specific force component. Nevertheless, this difference has a profound impact on the way these bacteria colonize a biomaterial surface.

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